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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
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International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IB 95/00265	18/04/95	29/04/94
Applicant		
AS BIOTEC-MACKZYMAL et al.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   [ ] filed with the international application.

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      [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

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4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

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   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

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(54) Title: ENZYME TREATMENT OF GLUCANS

(57) Abstract

β-(1-6)-Glucanase treatment of glucan from yeast cells, pure or feed grade, especially yeast from the family *Saccharomyces* and particularly *Saccharomyces cerevisiae*, provides a novel glucan product suitable for use in enhancing the stimulation of host animal immune systems. Solubilization of such yeast cell glucan is further disclosed to extend the usefulness of yeast cell glucan as an adjuvant.

ENZYME TREATMENT OF GLUCANS

This invention relates to the structural modification of yeast glucans, especially but not exclusively from the family Saccharomyces, by using ß-(1-6)-glucanase, and the use of such modified glucans in vaccine and animal feed formulations.

Background of the Invention

It is known from European Patent Application Ser.No. 91111-143.3 (Publication No. 0466031 A2) that the immune system of aquatic animals can be stimulated through the administering of an effective amount of a yeast cell wall glucan. It is further known that the effect of vaccines on such aquatic animals can be enhanced by the administering of an effective amount of such yeast cell wall glucan along with the vaccine antigens.

Such glucan compositions are particulate glucans such as that derived from the yeast Saccharomyces cerevisiae. Such particulate glucans are macromolecular and are comprised of a chain of glucose units linked by ß-(1-3)- and ß-(1-6)-linkages, said glucan being a branced β-(1,3)-glucan having β-(1,3)-linked and β-(1,6)-linked chains therein.

Such particulate glucans are provided by KS Biotec-Mackzymal under the brand "MacroGard" and are potent activators of the macrophage/monocyte cell series. Thus such particulate glucans have a profound effect on the immune system.

While the particulate glucan derived from Saccharomyces cerevisiae is recognized to have a variety of beneficial effects on fish and other animals, the use of the glucan in the particulate and thus insoluble form is limited.

In addition it is now believed that the presence of ß-(1-3)-branches contribute to the desired pharmaceutical benefits to be obtained from particulate glucan.

Accordingly a system whereby the ß-(1-3)-linked branches are made more readily available in the glucan would be highly desirable.

Summary of the Invention

In accordance with the present invention it has been discovered that by treating the particulate glucan derived from yeast organisms, especially of the family Saccharomyces, and particularly Saccharomyces cerevisiae, with a ß-(1-6)-glucanase, there is obtained a modified particulate glucan which is characterized by its enhanced activity in effecting stimulation of the immune system.

Thus in one embodiment of the present invention there is provided a novel ß-(1-3)-glucan from yeast which is characterized by its enhanced ability to stimulate the immune system of fish and other animals.

In another embodiment of this invention there is provided a novel process for the production of ß-(1-3)-glucan from yeast having enhanced pharmaceutical activity.

In another embodiment of this invention there is provided a novel solubilized ß-(1-3)-glucan from yeast which is useful for enhancing the activity of veterinary vaccines.

In still another embodiment of the present invention there is provided a novel feed grade glucan composition which is useful as an ingredient in conventional animal feeds.

Other embodiments and advantages of this invention will be apparent from the following specifications and claims.

Process for preparation of ß-(1-6)-glucanase treated glucan ("MacroGard").

"MacroGard" brand glucan is derived from Saccharomyces cerevisiae as disclosed in European Application Ser. No. 91111143.3. While such glucan is known to stimulate the immune system of fish, according to a preferred embodiment of the present invention, its activity is enhanced by the treatment thereof with a ß-(1-6)-glucanase.

Such glucanase treatment of the glucan is carried out by suspending the glucan particle in a buffered medium at a pH in the range of about 4 to about 8 and at a temperature in the range of from about 20 to about 50°C. Suitable buffered media are those selected from the group consisting of sodium acetate, ammonium acetate and sodium-potassium phosphate. Presently preferred buffer solutions are sodium acetate or ammonium acetate. Enzymatic degradation of the glucan is commenced by the addition of the ß-(1-6)-glucanase to the buffered medium.

ß-(1-6)-glucanases which are suitable for the modification of yeast glucan in accordance with the present invention are those obtained from a microorganism selected from the group consisting of Trichoderma longibrachiatum, Trichoderma reesei, Trichoderma harzianum, Rhizopus chinensis, Gibberella fujikuroi, Bacillus circulans, Mucor lilmalis, and Acinetobacter. Of these a presently preferred glucanase is that obtained from Trichoderma harzianum.

The amount of ß-(1-6)-glucanase employed for treatment of the glucan is normally in the range of from 1 to 50 U per g of glucan.

Enzymatic degradation is terminated by heating the reaction mixture to a temperature in the range of 80 to 100°C, preferably for a time in the range of 2 to 10 min. Other ways to

stop the enzyme degradation are, e.g. by adding proteases or inhibitors to the reaction mixture.

Alternatively the enzyme may be simply removed by washing. The washed particles are then resuspended in water with the addition of a bactericide such as 0.3% formalin (v/v) and stored at a temperature of about 4°C.

The resulting enzyme treated glucan can be characterized as a branched ß-(1-3)-glucan with ß-(1-3)-linked sidechains being attached by a ß-(1-6)-linkage and being essentially free of ß-(1-6)-linked chains. In this connection the phraze "β(1-6) chains" is meant to include branches of more than 1 β(1-6)-linked glucose units. The β-(1-6)-glucanase enzyme cleavage ensures that most chains of more than 4 β-(1-6)-bound glucose units are cleaved off.

To further enhance the utility of the glucan, it is subject to solubilization. Such solubilization treatment is generally carried out at a temperature in the range of about from 70 to 90°C for a period of from about 30 to 60 min in the presence of a solubilizing agent. A presently preferred solubilizing agent is formic acid. Following solubilization the solubilizing agent is removed and the resulting glucan is boiled in distilled water.

In practicing the present invention glucan can be first enzyme treated and then solubilized or conversely be solubilized and then enzyme treated.

In accordance with another embodiment of this invention there is provided a ß-(1-6)-glucanase treated feed grade glucan from yeast, e.g. Saccharomyces cerevisiae. Such feed grade glucan can be obtained by first contacting the yeast cell wall with an aqueous alkaline solution under conditions to effect the extraction of proteins and lipids therefrom. Generally such extraction is carried out at a temperature in

the range from about 50 to 80°C for about 2 to 8 h. A presently preferred alkaline extraction agent is sodium hydroxide. Following extraction, the cell walls are recovered from the aqeous alkaline solution and washed to remove solubilized cell wall components therefrom. The washed yeast cell wall are then neutralized by treatment with an acid such as phosphoric acid. Thereafter the neutralized washed glucan is pasteurized and then dried.

Suitable enzymes for treatment of the feed grade glucan are those useful in treating the high purity glucan.

The enzyme treated feed grade glucan is prepared by contacting the glucan with a ß-(1-6)-glucanase in the same manner as that employed to the enzyme treatment of the glucan particulate. The ß-(1-6)-glucanase treated feed grade glucan of this invention is useful in animal feed formulations.

The following examples are presented for purposes of illustration of the invention.

EXAMPLE 1

This example provides the protocol used to obtain an immunostimulatory glucan particle suitable for utilization in the practice of the present invention.

500 g of dry Saccharomyces cerevisiae was suspended in 3 l of 6 % aqueous NaOH solution. This suspension was then stirred overnight at room temperature. After stirring the suspension was centrifuged at 2000 x g for 25 min. The supernatant was discarded and the insoluble residue was then resuspended in 3 l of 3 % NaOH and incubated for 3 h at 75°C followed by cooling the suspension overnight. The suspension was then centrifuged at 2000 x g for 25 min and the supernatant was decanted. The residue was then resuspended in 3 % NaOH, heated and centrifuged as previously described.

The insoluble residue remaining was then adjusted to pH 4.5 with acetic acid. The insoluble residue was then washed with 2 l of water three times and recovered by centrifuging at 2000 x g for 25 min after each wash (the supernatant was poured off). The residue was then suspended in 3 l of a 0.5 M aqueous acetic acid. The suspension was heated for 3 h at 90°C. The suspension was then cooled to room temperature. After cooling, the insoluble residue was then collected by centrifuging at 2000 x g for 25 min. This treatment (from adjusting to pH 4.5 to collecting the cooled residue) was repeated 6 times.

The insoluble residue was then suspended in 3 l of distilled water and stirred for 30 min at 100°C, then cooled and centrifuged at 2000 x g for 25 min. The supernatant was discarded. The insoluble residue was washed in this manner 4 times. The residue was next suspended in 2 l of ethanol and heated at 78°C for 2 h. This wash with ethanol was repeated 4 times. The residue was then washed 4 times with 3 l of distilled water at room temperature to remove the ethanol, thereby

providing a suspension of desired glucan product.

EXAMPLE 2

This example provides the protocol to obtain glucan particles essentially free of ß-(1-6)-linked chains with the use of ß-(1-6)-glucanase isolated from Trichoderma harzianum.

200 mg of glucan particles prepared in accordance with Example 1 were suspended in 40 ml 50 mM ammonium acetate buffer, pH 5.0, together with 10 U of ß-(1-6)-glucanase at 37°C for 6h with constant stirring. The enzymatic degradation of the glucan particles was ended by heating the suspension at 100°C for 5 min. The particles were then washed three times with 200 ml sterile distilled H_2O by centrifugation at 2000 x g for 10 min, whereafter 185 mg of dried enzyme treated glucan was obtained.

The enzyme treatment will only cleave ß-(1-6)-linkages within ß-(1-6)-linked chains, but will not remove the ß-(1-6)-linked glucosyl residue extending from the branching points. The resulting enzyme treated glucan can be characterized as a branched ß-(1-3)-glucan with ß-(1-3)-linked sidechains being attached by a ß-(1-6)-linkage and being essentially free of ß-(1-6)-linked chains.

EXAMPLE 3

This example provides the protocol to solubilize glucan particles prepared in accordance with Example 1 by hydrolysis using formic acid (HCOOH).

2.0 g of glucan particles were suspended in 1.0 l of 90% formic acid and heated at 80°C for 45 min under constant stirring. The suspension was cooled to 35°C and the formic

acid was evaporated. The residue containing the hydrolysed particles was boiled in 500 ml distilled water for 3 h, whereafter the cooled suspension was filtrated through a 0.44 µm filter, frozen and lyophilized whereby 1.9 g of dry solubilized particles were obtained. The lyophilized solubilized particles were then dissolved in 100 ml distilled water and dialyzed, using a tubular dialysis membrane having a nominal molecular weight cut off (NMWCO) of 5000 Dalton, against tap water for 24 h, and then lyophilized. This resulted in 1.8 g solubilized glucan product.

EXAMPLE 4

This example demonstrates the biological effects of glucan particles prepared according to Example 1, and ß-(1-6)-glucanase treated glucan particles prepared according to Example 2 on immune responses in Atlantic salmon.

An A-layer positive isolate of Aeromonas salmonicida subsp. salmonicida, referred to as strain no. 3175/88 (Vikan Veterinary Fish Research Station, Namsos, Norway) was used. The bacterium was grown in brain heart infusion broth (Difco, USA) for 30 h at 14°C in a shaker incubator, and the culture medium with the bacterium was centrifuged for 10 min at 3000 x g. The pellet was resuspended in 0.9% saline, and the bacterium was killed by adding 0.5% formalin (v/v) to the suspension and incubating at 14°C for 24 h. The formalinized culture was then washed with sterile 0.9% saline and resuspended to a concentration of 2 x 10^9 ml^{-1} bacteria in 0.9% saline with 0.3% formalin. Bacterial suspensions were mixed with an equal volume of saline or with the different glucan suspensions (10 mg ml^{-1} in saline). Formalin was added to the vaccines to a final concentration of 0.3% (v/v).

In carrying out these experiments, two groups of experimental fish were used. In the vaccine experiment, Atlantic salmon

presmolts of 20 - 40 g were used. In the experiment where serum was collected for measuring blood lysozyme activity after glucan injection, Atlantic salmons of 50 - 70 g were used. The fish were kept in 150 l tanks supplied with aerated fresh water at 12°C and fed with commercial pellets ad libitum twice daily.

In the vaccination experiment 40 fish in each group were IP-injected with 0.1 ml of the different vaccine preparations or vaccine without glucan as a control. Blood was collected in evacuated tubes (Venoject, Terumo-Europe, Belgium) from 10 fish in each group 6, 10, and 18 weeks after injection. Blood samples were allowed to clot overnight at 4°C and sera were collected after centrifuging the tubes at 2000 x g for 10 min. Individual serum samples were transferred to Micronic serum tubes (Flow Laboratories Ltd., Lugano, Switzerland) and stored at -80°C until required.

In order to measure the effect of glucans on blood lysozyme activity, salmons were IP injected with 0.3 ml of the different glucans in saline or with 0.3 ml saline as the negative control. The glucans were administered at a concentration of 10 mg ml^{-1}. Blood samples were collected from 10 fish from each group 10 and 20 days after injection, using evacuated tubes (Venoject). The tubes were kept on ice until centrifuged at 2000 x g for 10 min, and individual serum samples were transferred to Micronic serum tubes and stored at -80°C until required.

Lysozyme activity was measured with the turbidimetric method using 0.2 mg ml^{-1} lyophilized Micrococcus lysodeikticus as the substrate in 0.04 M sodium phosphate buffer at pH 5.75. Serum (20 μl) was added to 3 ml of the suspension and the reduction in absorbance at 540 nm was measured after 0.5 min and 4.5 min at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. Results are expressed as mean lysozyme activity in serum from 10 fish (Tables 1 and

2).

The level of specific antibody against the A-layer of A. salmo-nicida in salmon sera was measured by an enzyme-linked immunosorbent assay (ELISA). A-layer protein was purified from whole A. salmonicida cells (Bjørnsdottir et al. (1992), Journal of Fish Diseases, 15:105-118), and protein content was determined (Bradford, M.M. (1976), Analytical Biochemistry, 72:248-254) using a dye-reagent concentrate from Bio-Rad Laboratories (Richmont, USA). Microtitre plates were coated with 100 μl of 5 μg ml^{-1} A-layer protein in 50 mM carbonate buffer, pH 9.6, and incubated overnight at 4°C. The further procedure was performed as described by Håvardstein et al. (Journal of Fish Diseases (1990), 13:101-111). The antibody titre in pooled serum samples was determined before individual serum samples were measured at three different dilutions (1:500, 1:1000 and 1:2000). Absorbance was read at 492 nm in a Multiscan MCC/340 MK II (Flow Laboratories Ltd). Results are expressed as mean antibody response to the A-layer of the bacterium at a dilution of 1:2000 in serum from 10 fish (Tables 1 and 2).

Table 1. Differences in biological effects of glucan particles and β-(1-6)-glucanase treated glucan particles on immune responses in Atlantic salmon.

	Saline control	Untreated glucan particles	β-(1,6)-glucanase treated glucan particles
Lysozyme activity post injection (units/ml)			
10 days	304	505	529
20 days	330	407	454

	Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with β-(1,6)-glucanase treated glucan particles
Antibody response post injection (absorbance)			
6 weeks	0.165	0.255	0.376
10 weeks	0.059	0.355	0.500
18 weeks	0.037	0.197	0.142

Both injection of untreated and β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.01$) lysozyme activity in serum compared to saline control both 10 and 20 days post injection. At day 20 post injection the lysozyme levels in fish injected with β-(1,6)-glucanase treated glucan particles were significantly higher ($p<0.05$) compared to fish injected with untreated particles.

β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.05$) antibody response to the vaccine compared to vaccine without adjuvant at all three sampling times, whereas untreated glucan particles induced significantly higher response 10 and 18 weeks post injection. β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.05$) antibody response than did untreated glucan particles at 10 weeks post injection, whereas no significant differences between the two were observed at 6 and 18 weeks post injection.

Table 2. Biological effects of glucan particles and solubilized glucan.

	Saline control	Untreated glucan particles	Solubilized glucan particles
Lysozyme activity (units/ml)			
10 days after injection	304	505	603
20 days after injection	330	407	773

	Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with solubilized glucan particles
Adjuvant effect (absorbance)			
6 weeks after injection	0.165	0.255	0.184
10 weeks after injection	0.059	0.355	0.349
18 weeks after injection	0.037	0.197	0.120

Injection of solubilized glucan particles induced significant higher ($p<0.01$) lysozyme activity than did untreated glucan particles both 10 and 20 days post injection. No significant differences could be observed between the ability of solubilized glucan particles and untreated glucan particles to

induce increased antibody response to the vaccine antigen at any sampling time point. Both induced significant higher ($p<0.05$) antibody response than vaccine without adjuvant 10 and 18 weeks post injection, but not at 6 weeks post injection.

EXAMPLE 5

This example provides the protocol to obtain a glucan composition suitable for use in the feeding of animals.

1000 kg of dry cell wall material of Saccharomyces cerevisiae was suspended in 5300 l of water at a temperature of 65°C in a stainless steel tank. To the suspension of cell walls in water there was added 227 l of 50% w/w NaOH so as to provide a caustic concentration of about 3%. The resulting mixture was then stirred for a period of about 4 h at a temperature of about 60°C.

Following the initial extraction period the suspension was then diluted with 8000 kg of water at a temperature of about 65°C in a stainless steel, stirred, washing tank such that the weight of the mixture was doubled. The resulting diluted mixture was then maintained at a temperature of about 60°C while being stirred for a period of about 15 min. Thereafter the resulting mixed diluted suspension was centrifuged in a nozzle centrifuge (Alfa Laval DX209). The supernatant was discarded. The resulting concentrated cell wall suspension was continuously introduced into a second steel stirred wash tank containing 8000 kg water and the mixture adjusted by the addition of water to give a final weight of 14500 kg. The resulting suspension was then mixed for a period of 15 min at a temperature of 60-65°C. Thereafter the agitated mixture was centrifuged.

The resulting cell wall suspension was continuously added to

a third vessel containing 8000 kg water. Additional water at 60°C was added to provide a final weight of 14500 kg. The resulting suspension was stirred for a period of 15 min at 60-65°C and thereafter centrifuged.

Following centrifugation, the resulting cell wall concentrate was transferred to a stainless steel storage tank wherein the suspension was cooled to a temperature of about 5-10°C. The resulting cooled suspension was treated with phosphoric acid (H_3PO_4) in a stainless steel agitated tank in an amount to achieve a suspension of solids having a pH of 5.5-7.5.

Following neutralization the resulting neutralized mixture was subjected to pasteurization by heating at a temperature of 75°C for a period of 18 seconds by passing the mixture through an in-line plate and frame heat exchanger.

Following pasteurization the resulting pasteurized mixture was then spray dried in a spray drier maintained at an inlet air temperature of at least 140-150°C and an exhaust temperature of about 65-70°C whereby there was achieved 300 kg of dry glucan product.

EXAMPLE 6

This example provides the protocol and effect of treatment of feed grade glucan with a ß-(1-6)-glucanase.

25 g of feed grade glucan, prepared in accordance with Example 5, suspended in 1.25 l of 50 mM sodium acetate, pH 5.0, in a 2 l conical flask. Glucan particles were maintained in suspension by shaking, the suspension was warmed to 30°C and purified ß-(1-6)-glucanase from Trichoderma harzianum was added to a final concentration of 1.8 U/g glucan.

To follow the timecourse of the enzymatic removal of ß-1,6-

bound glucose from the glucan particle, 1 ml aliquotes of the suspension were withdrawn at different timepoints, centrifuged at 2000 x g, and 0.2 ml of the supernatants analyzed for free, reducing carbohydrate (Nelson et al. (1944), Journal of Biological Chemistry, 153:315-80). The glucan suspension was incubated for 28 h at which time the rate of release of free, reducing carbohydrate was observed to be very low. The glucan particles were then pelleted by centrifugation at 2000 x g, washed once in 50 mM sodium-acetate, pH 5.0 and once in water.

A fine, dry powder suitable for use as a feed additive was prepared from the wet glucan by first dehydrating the pellet four times with ethanol at room temperature followed by air drying at room temperature.

Results from treating a feed grade glucan with ß-(1-6)-glucanase from T. harzianum as described above are shown in Table 3.

Table 3. Liberation of glucose from feed grade glucan during treatment with β-(1-6)-glucanase from T. harzianum.

Enzyme reaction time, [h]	Glucose liberated, [% of total glucose in glucan]
0	0.0
0.5	1.9
1	2.6
2	3.3
3	3.7
4	4.0
5	4.3
2	5.5
8	5.6

C l a i m s

1. A process for the preparation of a glucan product from yeast which comprises:

(a) contacting a branched ß-(1-3)-glucan having ß-(1-3)-linked and ß-(1-6)-linked chains therein with a ß-(1-6)-glucanase under conditions such that the resulting glucan is comprised of ß-(1-3)-linked glucose units and is essentially free of ß-(1-6)-linked chains.

2. A process according to claim 1 wherein said ß-(1-6)-glucanase is obtained from the groups of microorganisms consisting of Trichoderma longibrachiatum, Trichoderma reesei, Trichoderma harzianum, Rhizopus chinensis, Gibberella fujikuroi, Bacillus circulans, Mucor lilmalis and Acinetobacter.

3. A process in accordance with claim 1 wherein said ß-(1-6)-glucanase is obtained from Trichodermia harzianum.

4. The process of claim 1 wherein the particulate ß-(1-3)-glucan is derived from yeast of the family Saccharomyces.

5. The process of claim 4 wherein the particulate β-(1,3)-glucan is derived from Saccharomyces cerevisiae.

6. The process of claim 1 wherein said insoluble particulate ß-(1-3)-glucan is prepared by the process comprising:

(a) alkali-extracting suitable glucan-containing yeast cells with a suitable extractive aqueous alkali solution under suitable conditions to provide a first insoluble yeast residue.

(b) hot alkali-extracting said first insoluble yeast residue

with a suitable extractive aqueous alkali solution under suitable extraction conditions wherein the hot alkali extraction is performed at least 2 times to provide a second insoluble yeast residue and recovering the insoluble yeast residue after hot alkali extraction; thereafter

(c) washing said second insoluble yeast residue with a suitable hydrolyzing acid under suitable conditions with water at a pH in the range of from about pH 4 to about pH 7 thereby providing a third insoluble yeast residue and recovering said third insoluble yeast residue after the wash;

(d) hydrolyzing said third insoluble yeast residue under mild acidic hydrolysis condition wherein the acid hydrolysis is performed at least 3 times to provide a fourth insoluble yeast residue and recovering the yeast residue after each acid hydrolysis; thereafter

(e) boiling said fourth insoluble yeast residue under suitable conditions in water wherein the boiling of said fourth insoluble yeast residue is performed at least 2 times to provide a fifth insoluble yeast residue and recovering the insoluble yeast residue after each boiling; and

(f) boiling said fifth insoluble yeast residue under suitable conditions in ethanol wherein the boiling in ethanol of said fifth yeast residue is performed at least 2 times to provide a sixth insoluble yeast residue and recovering the insoluble yeast residue after each boiling; thereafter

(g) washing said sixth insoluble yeast residue under suitable conditions with water wherein the washing of said sixth yeast residue is performed at least 2 times to provide a yeast glucan and recovering the insoluble yeast residue after each wash.

7. The product of the process of claim 1, being characterized as a branched ß-(1-3)-glucan with ß-(1-3)-linked sidechains being attached by a ß-(1-6)-linkage and being essentially free of ß-(1-6)-linked chains.

8. The product of the process of claim 6, being characterized as a branched ß-(1-3)-glucan with ß-(1-3)-linked sidechains being attached by a ß-(1-6)-linkage and being essentially free of ß-(1-6)-linked chains.

9. An insoluble particulate yeast glucan especially from the yeast family Saccharomyces and particularily from the yeast species Saccharomyces cerevisiae being characterized as a branched ß-(1-3)-glucan with ß-(1-3)-linked sidechains being attached by a ß-(1-6)-linkage and being essentially free of ß-(1-6)-linked chains.

10. A process for the production of a solubilized ß-(1-3)-glucan particle from yeast, especially from the yeast family Saccharomyces and particularily from the yeast species Saccahromyces cerevisiae, which comprises contacting an insoluble glucan from the yeast family Saccharomyces having a backbone of ß-(1-3)-linked glucose units with at least one ß-(1-3)-linked side chain of at least 1 glucose units attached thereto with a solubilizing agent.

11. A process in accordance to claim 10 wherein said solubilizing agent is formic acid and said insoluble glucan is contacted with said solubilizing agent at a temperature in the range of from 70 to 90°C.

12. The solubilized ß-(1-3)-glucan product of the process of claim 11.

13. A process for the preparation of a feed glucan product from yeast, especially from the yeast family Saccharomyces and particularily from the yeast species Saccahromyces

cerevisiae, which comprises:

(a) contacting the feed grade yeast glucan being a branched ß-(1-3)-glucan having ß-(1-3)-linked and ß-(1-6)-linked chains therein with a ß-(1-6)-glucanase under conditions such that the resulting glucan is comprised of ß-(1-3)-linked glucose units and is essentially free of ß-(1-6)-linked chains.

14. The process of claim 13 wherein said glucan is derived from Saccharomyces cerevisiae.

15. The process of claim 14 wherein said feed grade glucan is prepared by the process comprising:

(a) contacting yeast cell walls with an aqueous alkaline solu-tion under suitable conditions to effect the extraction of proteins and lipids therefrom;

(b) separating the resulting extracted yeast cell walls from said aqueous alkaline solution;

(c) washing the resulting separate yeast cells so as to further remove solubilized cell wall components therefrom;

(d) neutralizing the washed yeast cell walls; and

(e) pasteurizing the neutralized, washed cell walls and thereafter drying the resulting pasteurized, neutralized, washed cell walls.

16. The product of the process of claim 13, being characterized as a branched ß-(1-3)-feed grade glucan with ß-(1-3)-linked sidechains being attached by a ß-(1-6)-linkage and being essentially free of ß-(1-6)-linked chains.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 028 703 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 2 July 1991 see column 2, line 48 - column 3, line 50 see claims 1-15 ---	1-6, 10-16
X	DATABASE WPI Section Ch, Week 7949 Derwent Publications Ltd., London, GB; Class B04, AN 79-88295B & JP,A,54 138 115 (KIRIN BREWERY KK) , 26 October 1979 see abstract --- -/--	7-9,16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 1995	03. 10. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Douschan, K

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS LETTERS, vol. 64, no. 1, 1976 pages 44-47, S.BALINT ET AL. 'Biosynthesis of Beta-Glucans catalyzed by a particulate enzyme preparation from yeast' see the whole document	7-9,16
X	J. BIOCHEM., vol. 98, no. 5, 1985 pages 1301-1307, MASAO SHIOTA ET AL. 'Comparison of Beta-Glucan structures in a cell wall mutant of Sacch. cerevisiae and the wild type' see the whole document	7-9,16
P,X	DEV. COMP. IMMUN., vol. 18, no. 5, 1994 pages 397-408, R. ENGSTAD, B. ROBERTSEN 'Specificity of a Beta-Glucan Receptor on macrophages from atlantic salmon' see the whole document	1-16
A	EP,A,0 466 037 (PHILLIPS PETROLEUM COMP.) 15 January 1992 cited in the application see claims 1-6	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 95/00265

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5028703	02-07-91	US-A-	5250436	05-10-93
EP-A-0466037	15-01-92	AU-B-	628752	17-09-92
		AU-A-	7933891	23-01-92
		CA-A-	2040374	07-01-92
		JP-A-	4253703	09-09-92
		US-A-	5401727	28-03-95

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PATENT COOPERATION TREATY

PCT

REC'D 30 JUL 1996
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
94.1581		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB 95/00265	18/04/1995	29/04/1994
International Patent Classification (IPC) or national classification and IPC		
C12P19/14		
Applicant		
AS BIOTEC-MACKZYMAL et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36:

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of _____ sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/11/1995	25.07.96
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	K. Douschan Telephone No.

Form PCT/IPEA/409 (cover sheet) (January 1994) (08/01/1996)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

[x] the international application as originally filed.

[ ] the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____,

[ ] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,
Nos. _____, filed with the letter of _____,

[ ] the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:
[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-6, 13-15: YES	YES
	Claims 7-12, 16: NO	NO
Inventive Step (IS)	Claims 1-16: NO	YES
	Claims 1-16: NO	NO
Industrial Applicability (IA)	Claims 1-16: YES	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

1). The documents mentioned in the International Search Report are cited by the following abbreviations:
D1: US-A-5 028 703,
D2: DATABASE WPI, Section Ch, Week 7949, Class B04, AN 79-88295B,
D3: FEBS letters, vol. 64, no. 1, 1976, p. 44-47,
D4: J. BIOCHEM., vol. 98, no. 5, 1985, p. 1301-1307,
D5: DEV. COMP. IMMUN., vol. 18, no. 5, 1994, p. 397-408,
D6: EP-A-0 466 037.

2). The priority documents pertaining to the present application were not available at the time of establishing this International Preliminary Examination Report. Hence, it is based on the assumption that all claims enjoy priority rights from the filing date of the priority document. If it later turns out that this is not correct, the document D5 cited in the international search report could become relevant to assess whether claims 1-16 satisfy the criteria set forth in Article 33(1) PCT.

3). Document D1 discloses a method for altering ß-(1-6)-linkages of a glucan derived from yeast, comprising treating the said glucan with ß-(1-6)glucanase. Moreover, D1 discloses a change of the solubilisation profile of the glucans after acid treatment.
D2, D3 and D4 cite glucans from yeast which consist of "mainly ß-(1-3)glucoside bonds.
D5 is not to be taken into consideration at this stage of the examination procedure (see point 2 above).
D6 discloses how to obtain the starting compounds, i.e. the particulate glucans from yeast, and corresponds to present claim 6.

4). **Novelty (Art. 33(1) and (2) PCT):**
The compounds claimed in present claims 7-9 and 16 lack novelty in the light of documents D2, D3 and D4, since the present wording "essentially free of ß-(1-6)-linked chains" does not sufficiently delimit the claimed compounds from D2, D3 and D4 which use similar wordings.

The solubilisation process and the solubilized product claimed in claims 10-12 of the present patent application is not novel in the light of D1, where the solubilisation properties of glucans derived from yeast are changed by means of acid (with regard to claims 10-12 see also item VIII of this communication).

The processes claimed in claims 1-6 and 13-15 appear to be novel since D1 uses the ß-(1-6) treatment of the glucans to alter the ß-(1-6) linkages, not to destroy them.
D4 performs the treatment with the ß-(1-)-glucanase at the end of the process for explore the structure of the resulting compounds, and therefore does not destroy the novelty of claims 1-6 and 13-15.

5). Inventive Step (Art. 33(1) and (3) PCT):

None of the subject-matter claimed in claims 1-16 involves an inventive step, since it is either known or obvious from the prior art.

The process claimed in claims 1-6 and 13-15 cannot be seen as inventive in the light of D1. D1 discloses a method of altering the ß-(1-6)-linkages of glucans by treating a glucan derived from yeast with a ß-(1-6)-glucanase. Although it is assumed that the altered linkages of the glucans mentioned in D1 do not correspond to the destroyed linkages of the present glucans, the claimed process nevertheless does not involve an inventive step in the light of D1.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

a) To meet the requirements of Rule 5.1(a)(ii) PCT, the documents D1-D4 should be identified in the description and the relevant background art disclosed therein should be briefly discussed.

b) The number "EP-A-0 466 031" cited on p. 1 of the description should read "EP-A-0 466 037" (see International Search Report).

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

a) It is stressed that the claims in their present form will result in a non-unity-objection under Rule 13 PCT at a later stage of the proceedings, since the process of claim 10 (and dependent claims 11 and 12) do not contain a reference to an earlier claim, thus resulting in subject-matter which is not linked with the subject-matter of the other claims as to form one single inventive concept.
The problem underlying the present invention is to provide a process for the production of pure ß-(1-3)-linked glucans from yeast-derived glucan (containing also ß-(1-6)-linkages). Claims 10-12 are not linked with this problem, but concern the solubilisation of glucans which are broader defined (they are defined merely as containing ß-(1-3)-linkages, the absence of (1-6)-linkages is not mentioned).
Since it is assumed that the solubilisation of glucans derived from the other processes of the present invention is meant (see p. 5 of the present description), an objection under Rule 13 PCT is not yet raised.

b) Some of the features in the process claim 1 relate to the object and/or result to be achieved by the said process ("functional feature") rather than imposing any clear instructions how to achieve the said results. To meet the requirements of Article 6 PCT, the claim should be recast to make the intended limitations clear.
Moreover, D1 renders claim 1 and the "functional feature" therein obscure since the treatment of yeast-derived glucans with ß-(1-6)-glucanase results in "altered" (1-6)-linkages in the process of D1, whereas it destroys the said linkages in the present process. As

long as the product resulting from the claimed process is different from that of D1, the functional feature in claim 1 has to be specified.

c) The claims in their present wording lack clarity for the following reasons (Art. 6 PCT):
- it has to be made clear whether the starting compound is a branched and/or particulate glucan;
- the term "said insoluble particulate ..." is not clear since this term has not been used in either of claims 1-5;
- it is not clear in claims 6 and 15 which reaction step is performed.

d) Claims 6 and 15 appear to have no basis in the description (Art. 6 PCT).

e) Claims 7, 8, 12 and 16 are drafted as "product-by-process"-claims. It is stressed that this wording means under e.g. the EPC, that the product per se is claimed and has to fulfil the requirements for novelty and inventive step.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Washington D.C. 20231
United States of America

in its capacity as elected Office

Date of mailing (day/month/year) 08 January 1996 (08.01.96)	
International application No. PCT/IB95/00265	**Applicant's or agent's file reference** 94.1581
International filing date (day/month/year) 18 April 1995 (18.04.95)	**Priority date** (day/month/year) 29 April 1994 (29.04.94)
Applicant ENGSTAD, Rolf et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

20 November 1995 (20.11.95)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

H. Zhou

Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
KEEFER, Timothy, J.
LADAS & PARRY
224 South Michigan Avenue
Suite 1200
Chicago, Illinois 60604
ETATS-UNIS D'AMERIQUE

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	25.07.96
Applicant's or agent's file reference: 94.1581	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB 95/00265	18/04/1995	29/04/1994

Applicant

AS BIOTEC-MACKZYMAL et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Naomi Hardy Magliano Telephone No.